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ORIGINAL PAPER

JAB1 accelerates odontogenic differentiation of dental pulp stem cells

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Abstract Jun activation domain-binding protein 1 (JAB1) is a multifunctional protein that participates in the control of cell proliferation and the stability of multiple proteins. JAB1 regulates several key proteins, and thereby produces varied effects on cell cycle progression, genome stability and cell survival. Some studies have shown that the loss of JAB1 in osteochondral progenitor cells severely impairs embryonic limb development in mice. However, the biological significance of JAB1 activity in the odontogenic differentiation of dental pulp stem cells (DPSCs) remains unclear. This study aimed to determine the role of JAB1, a key player in tooth development, in reparative dentin formation, especially odontogenic differentiation. We found that increased expression of JAB1 promoted odontogenic differentiation of DPSCs via Wnt/β-catenin signaling. The role of JAB1 in the odontogenic differentiation of DPSCs was further confirmed by knocking down JAB1. Our findings provide novel insights on odontogenic differentiation of DPSCs.

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Keywords Jun activation domain-binding protein 1 (JAB1) · Dental pulp stem cells (DPSCs) · Odontogenic differentiation · Wnt/β-catenin signaling

Introduction

Dental pulp tissue is easily damaged by bacterial infections, mainly through dental caries and traumatic injuries (Yu and Abbott 2007). Currently, root canal therapy is the only clinical treatment available for damaged or necrotic dental pulp tissue arising from caries, but results in loss of tooth vitality. Somatic dental stem cells-based tissue engineering approaches can alleviate this problem by preserving tooth vitality (Ravindran et al. 2014a, b). The traumatized dental pulp can regenerate tertiary dentin by differentiating into odontoblast-like cells (Tziafas et al. 2001; Rajendran et al. 2013). The aim of vital pulp therapy is to maintain pulp vitality and function (Zhao et al. 2012). Previous studies have indicated that intense stimuli, such as cavity preparation and advanced dental caries, may cause the death of odontoblasts and stimulate odontogenic differentiation of dental pulp stem cells (DPSCs) followed by reparative dentin formation (Lin et al. 2011; Du et al. 2016).

DPSCs, a unique group of cells with clonogenic ability, high reproductive activity and multiple differentiation potentials, were demonstrated to be crucial for tertiary dentinogenesis (Gronthos et al. 2000; Patel et al. 2009). DPSCs are capable of both self-renewal and multi-lineage differentiation (Gronthos et al. 2002; Ferro et al. 2014; Park et al. 2015). The most notable characteristic of DPSCs is to regenerate dentin–pulp-like complexes (Gronthos et al. 2002; Alge et al. 2010; Song et al. 2015).

Understanding the mechanisms that regulate odontoblastic differentiation in DPSCs will have significant implications for the development of new therapeutic strategies in dental pulp injury.

Jun activation domain-binding protein 1 (JAB1) was primitively identified as a transcriptional co-activator of c-Jun protein by stabilization of the activator protein 1 complex, resulting in increased specificity of target gene activation (Bech-Otschir et al. 2001; Wan et al. 2002; Tian et al. 2010). JAB1 is critical for the functional inactivation of several key negative regulatory proteins in cellular proliferation through their subcellular localization, degradation, phosphorylation and deneddylation (Oh et al. 2006; Wang et al. 2015). JAB1 is also the fifth component of the COP9 signalosome (CSN) complex (COPS5), which is involved in various cellular and developmental processes (Bae et al. 2002; Wei and Deng 2003; Martin and Wang 2015). JAB1 could activate related transcriptional factors, which contribute to cell proliferation and differentiation (Xu et al. 2015). The transcriptional co-regulator JAB1 was also shown to be crucial for chondrocyte differentiation in vivo (Chen et al. 2013). Furthermore, knockdown of CSN5/JAB1 led to reduced β-catenin and phospho-β-catenin levels in colorectal cancer cells (Schutz et al. 2012). However, the role of JAB1 in the odontogenic differentiation of DPSCs remains unclear.

In recent years, numerous trials have shown that the canonical Wnt pathway is crucial for differentiation in many types of stem cells (Ahmadzadeh et al. 2015). A previous study has found that Wnt/β-catenin signaling pathway is partly responsible for Berberine-induced osteogenic differentiation of mesenchymal stem cells (MSCs) in vitro (Tao et al. 2016). JAB1 is thought to be critical for the regulation of β-catenin levels and the associated Wnt signaling in colorectal cancer cells (Schutz et al. 2012; Qin et al. 2015). Our previous study also confirmed that Wnt/β-catenin signaling pathway is involved in osteogenic differentiation of DPSCs (Feng et al. 2015). However, how JAB1 regulates the odontogenic differentiation of DPSCs remains controversial.

In this study, we investigated the expression and localization of JAB1 in DPSCs, and its effect on the odontogenic differentiation of DPSCs. We found that JAB1 knockdown decreased odontogenic differentiation of DPSCs and expression of nuclear β-catenin in vitro. Furthermore, suppression of β-catenin by DKK-1 inhibited odontogenic differentiation DPSCs. These results provide new insights into the mechanisms of JAB1 and suggest that Wnt/β-catenin could be the key pathway associated with the odontogenic differentiation of DPSCs.

Materials and methods

Cell culture

Normal human impacted third molars were collected from patients 13–23 years of age (n = 9) after giving the informed consents which were approved by the Ethics Committee of the Affiliated Hospital of Nantong University with the following reference number 2015-018. All subjects were free of carious lesions and oral infection. We isolated DPSCs by cleaning the tooth surface, cutting around the cemento-enamel junction using sterilized dental fissure burs and then opening to reveal the pulp chamber. The pulp was then digested in a solution of 3 mg/ml collagenase type I for 1 h at 37 °C. Single-cell suspensions were obtained by passing the digested tissues through a 70-μm cell strainer (BD Falcon). Cell suspensions of dental pulp were seeded into 25 cm^2 culture dishes and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5 % CO_2. The medium was changed every 3 days. Approximately 7–10 days after seeding, the cells became nearly confluent. Cells were passaged at the ratio of 1:3 when they reached 85 % to 90 % confluence. The adherent cells were released from the dishes with 0.25 % trypsin (Gibco, USA) and seeded into new fresh culture flasks. All the experiments described below were performed using DPSCs from the mixed population of cells at passage 3 (P3). The cell populations positively expressed STRO-1, CD34 and c-kit, while negatively expressing CD45 (Feng et al. 2013).

Odontogenic differentiation

Third passage DPSCs (2×10^4 cells/dish) were seeded in 35 mm culture dishes (Costar, Cambridge, MA) and cultured with odontogenic differentiation induction medium consisting of α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA,USA) supplemented with 15 % fetal bovine serum (FBS; Gibco-BRL, Life Technologies Inc, Gaithersburg, MD, USA), 50 mg/mL α-ascorbic acid, 10 mmol/L β-glycerophosphate, 10 nmol/L dexamethasone (Sigma-Aldrich, St Louis, MO) and 0.292 mg/mL glutamine, 100 U/mL penicillin G, 100 mg/mL streptomycin respectively for 14, days, replacing the medium every 2 days. After induced for 14 days, cells were prepared for immunofluorescence and alizarin red S staining. RNA and protein were extracted for real-time RT-PCR, Western blot analysis.

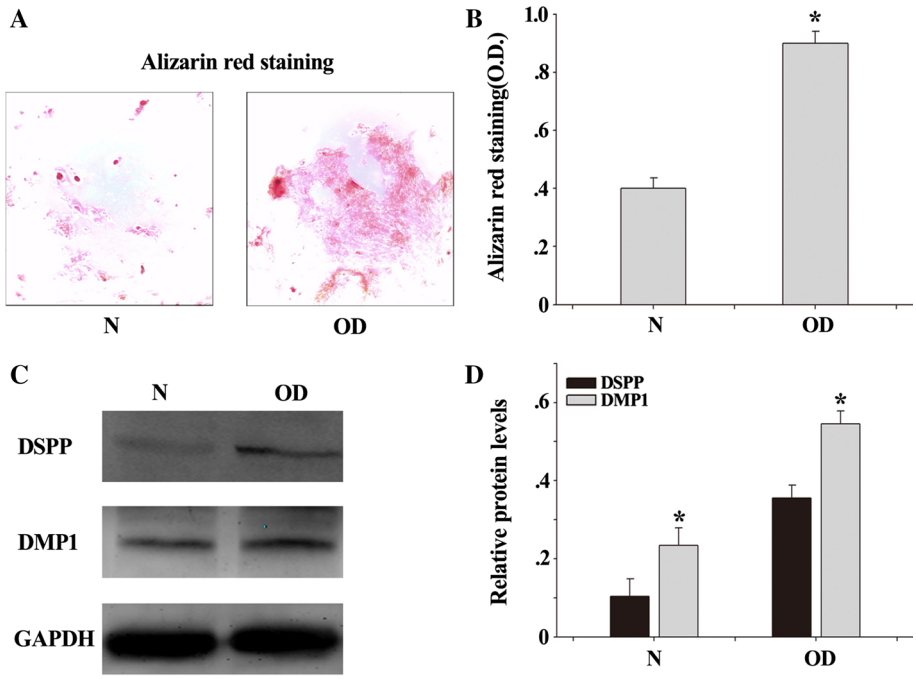

Fig. 1 The mineralization and proliferation abilities of DPSCs during odontogenic differentiation. **a** DPSCs were cultured in odontogenic differentiation medium for 14 days and stained with Alizarin red S. *OD* odontogenic differentiation, *N* normal culture. **b** Quantification of Alizarin red S staining. $*P < 0.05$. **c** The expression of DSPP and DMP1 proteins were analyzed by Western blot. GAPDH expression was determined as a control. **d** Quantification of DSPP and DMP1 protein levels.$*P < 0.05$

Alizarin red S and staining

DPSCs were fixed with 4 % PFA for 1 h and washed with PBS. Cells were then stained with 40 mmol/L alizarin red S (pH 4.2) for 10 min under conditions of gentle agitation. After destaining and air-drying, culture plates were evaluated by light microscopy using an inverted microscope. To quantify the red dye, the stain was solubilized by shaking with 10 % cetylpyridinum chloride for 20 min and absorbance was measured at 570 nm.

Western blot

Cells were lysed in the buffer consisting of 50 mM TRIS, 150 mM NaCl, 2 % sodium dodecyl sulfate (SDS) and a protease inhibitor mixture. After centrifugation at 12,000 rpm for 12 min, protein concentrations were determined using the Bradford assay (Bio-Rad). The resulting supernatant (50 μg of protein) was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred onto PVDF membranes at 350 mA for 2.5 h in a blotting apparatus (Bio-RAD, CA, USA). The membrane was blocked with 5 % milk in PBST for 1 h at room temperature (RT). The samples were incubated with the primary antibody overnight at 4 °C and the HRP-conjugated secondary antibodies for 2 h. GAPDH and β-actin were used as the internal control for the cytoplasmic and nuclear proteins. The following primary antibodies were used: GAPDH (anti-rabbit, Santa Cruz), DSPP (antirabbit, Santa Cruz), DMP1 (anti-rabbit, Santa Cruz), JAB1 (anti-rabbit, Santa Cruz), β-actin (anti-mouse, Santa Cruz), β-catenin (anti-mouse, Cell Signaling), Lamin B (anti-mouse, Santa Cruz), GSK-3β (anti-mouse; Santa Cruz).

Immunofluorescent staining

DPSCs were fixed with 4 % PFA for 1 h, washed with PBS containing 0.1 % Triton X-100 (PBST), and blocked for 30 min in PBST supplemented with 10 % FBS. Cells were then incubated with one of the following primary antibodies (1:100) in the same solution overnight at 4 °C: JAB1 (anti-rabbit, Santa Cruz), β-catenin (anti-mouse, Cell Signaling). Cells were then washed and incubated with secondary antibodies for 2 h at room temperature. Nuclei were stained with DAPI (4′6′-diamidino-2-phenylindole dihydrochloride) (1:800; Santa Cruz). The cells were examined using a Leica fluorescence microscope (Germany).

siRNAs and transfection

siRNA transfection was carried out using a commercially available kit (GENECHEM). For siRNA inhibition studies, DPSCs were washed with the siRNA transfection medium and then incubated (at 37 °C and 5 % CO_2) for 12 h with transfection medium containing the transfection reagent and either JAB1 siRNA (50 nmol/L) or control siRNA (50 nmol/L), according to the manufacturer's instructions. After transfection, the cells were harvested at 72 h for RNA or protein extraction.

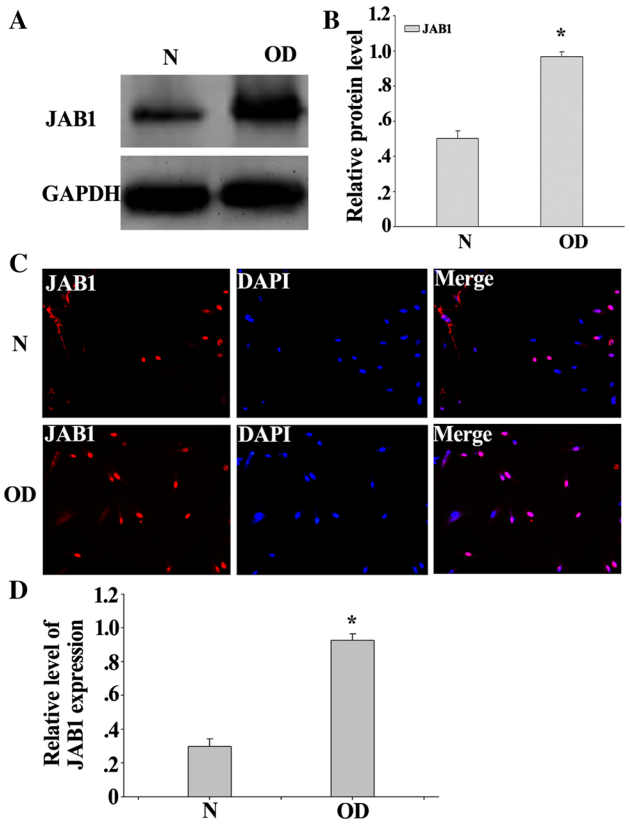

Fig. 2 Increased expression of JAB1 during the odontogenic differentiation of DPSCs. **a** JAB1 expression was analyzed by Western blot after the cells were cultured in odontogenic differentiation medium for 14 days. **b** Quantification of JAB1 protein level. $*P < 0.05$. **c** Immunocytochemistry of JAB1. *Blue*, DAPI. *Scale bar* 25 μm. **d** Quantification of JAB1-positive cells. The Sirt1-positive cell ratio was counted by phase-contrast microscopy $*P < 0.05$. (Color figure online)

Real-time RT-PCR

Total RNA was extracted from cells and reverse transcribed using conventional protocols. PCR amplification was performed using the following primer sets: GAPDH 5′-TCCATGACAAC-TTTGGTATCG-3′, 5′-TGTAGCCA AATTCGTTGTCA-3′; DSPP 5′-GGAGACAAGACCTCC AAGAGTA-3′, 5′-TGCTGGGACCCTTGATTTCTA-3′; DMP1 5′-TGGGGATTATCCTGTGCTCT-3′, 5′-GCTGTC ACTGGGGTCTTCA T-3′; JAB1 5′-ATAGATCTATGG CGGCTTCTGGGAG-3′, 5′-TAGGGCCCTTAAGAGATG TTAATTTG-3′. All the primer sequences were determined using established GenBank sequences. The primers were used to amplify the duplicate PCR reactions. Each sample was analyzed in triplicate and GAPDH was used as a control.

Statistical analysis

The data were analyzed and expressed as the mean ± standard deviation. The significance of differences between the experimental groups and controls was analyzed using ANOVA. Statistical significance was evaluated by the independent samples *t* test using SPSS v17.0 software. Values of $P < 0.05$ were regarded as significant.

Results

The mineralization ability of DPSCs during odontogenic differentiation

To investigate the odontogenic differentiation process of DPSCs, we measured the formation of mineralized nodules and expression of odontoblast-related marker genes. The mineral nodules were not detected until day 14 by alizarin red S staining (Fig. 1a, b). The level of DSPP and DMP1 increased significantly on day 14 (Fig. 1c, d). These results revealed increased odontogenic differentiation ability of DPSCs after 14 days of induction.

Expression of JAB1 during odontogenic differentiation of DPSCs

Recent research has shown that JAB1 is associated with cell differentiation. Western blotting showed that JAB1 protein level increased during odontogenic differentiation of DPSCs (Fig. 2a, b). Immunofluorescence results also confirmed an obvious upregulation of JAB1 on day 14 after odontogenic induction (Fig. 2c, d). The above results indicated that the expression of JAB1 increased after induction.

Knockdown of JAB1 inhibits odontogenic differentiation of DPSCs

To determine the role of JAB1 in the odontogenic differentiation of DPSCs, we used siRNA to silence JAB1 expression. Western blot and RT-PCR were performed to assess the silencing effects of siRNA on JAB1. We found that transfection with siRNA led to significantly decreased expression of JAB1 in cells (Fig. 3a–c). Western blot and RT-PCR showed that siRNA-mediated silencing of JAB1 resulted in decreased expression of DSPP and DMP1 (Fig. 3d, e). The above results showed that siRNA-mediated silencing of JAB1 expression could inhibit odontogenic differentiation of DPSCs.

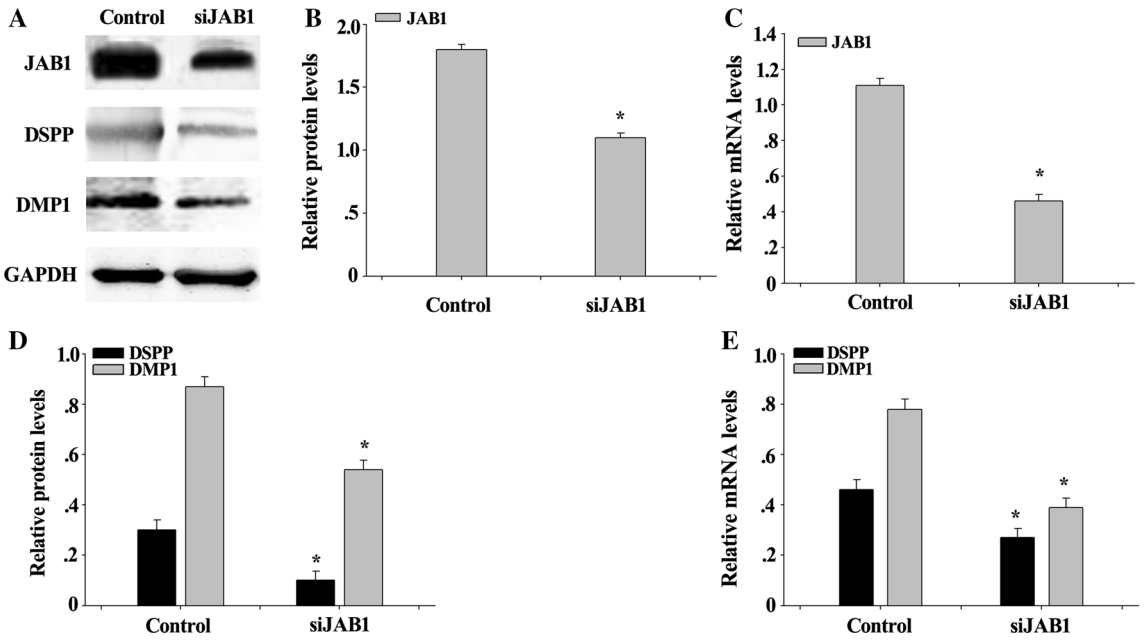

Fig. 3 Knockdown of JAB1 inhibits odontogenic differentiation of DPSCs. **a** siRNA was used to silence JAB1 expression. The expression of JAB1, DSPP and DMP1 proteins were detected by Western blot. **b** Quantification of JAB1 protein levels. $*P < 0.05$. **c** Quantitation of JAB1 mRNA levels. The quantity of amplified product was analyzed by an image analyzer. $*P < 0.05$. **d** Quantification of DSPP and DMP1 protein levels. $*P < 0.05$. **e** Quantitation of DSPP and DMP1 mRNA levels. The quantity of amplified product was analyzed by an image analyzer. $*P < 0.05$

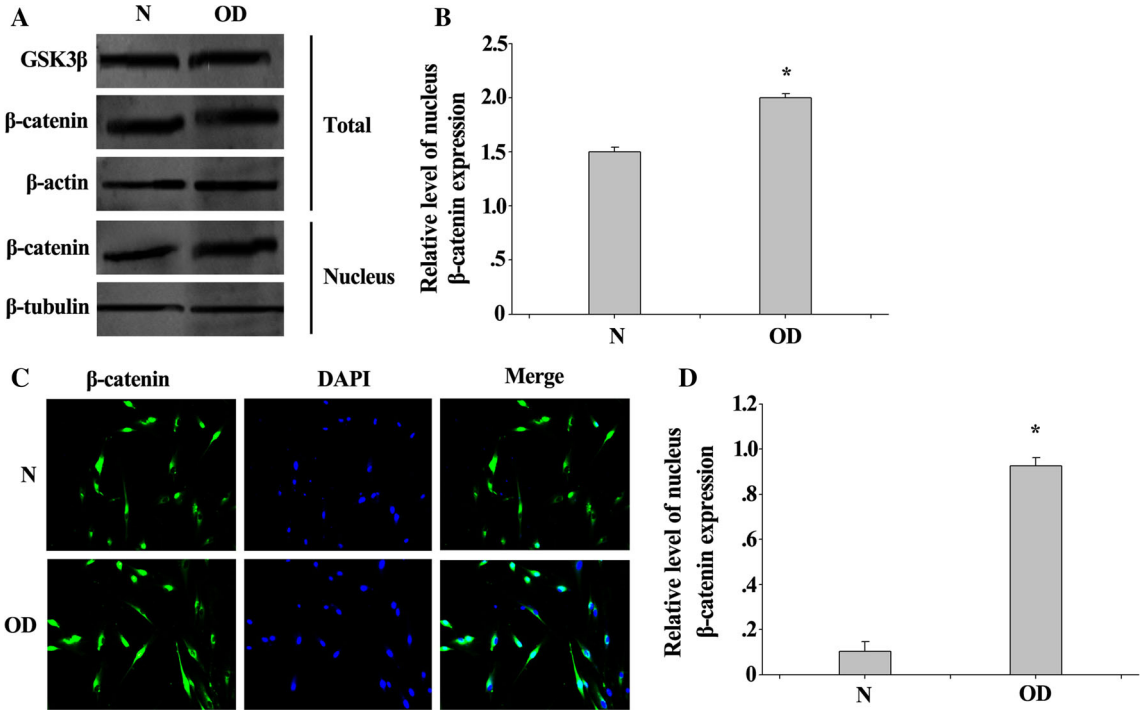

Fig. 4 Activation of Wnt/β-catenin signaling pathway in odontogenic differentiation of DPSCs. **a** DPSCs were cultured in odontogenic differentiation medium for 14 days. The total and nuclear β-catenin levels were analyzed by Western blot. β-actin was used as the internal control for cytoplasmic proteins. **b** Quantification of nuclear β-catenin level. $*P < 0.05$. **c** Immunocytochemistry of β-catenin. *Blue*, DAPI. In the OD group, β-catenin expression in the cytoplasm was clearly increased as compared to the normal group. *Scale bar* 25 μm. **d** Quantification of β-catenin- positive cells. The Sirt1-positive cell ratio was counted by phase-contrast microscopy $*P < 0.05$. (Color figure online)

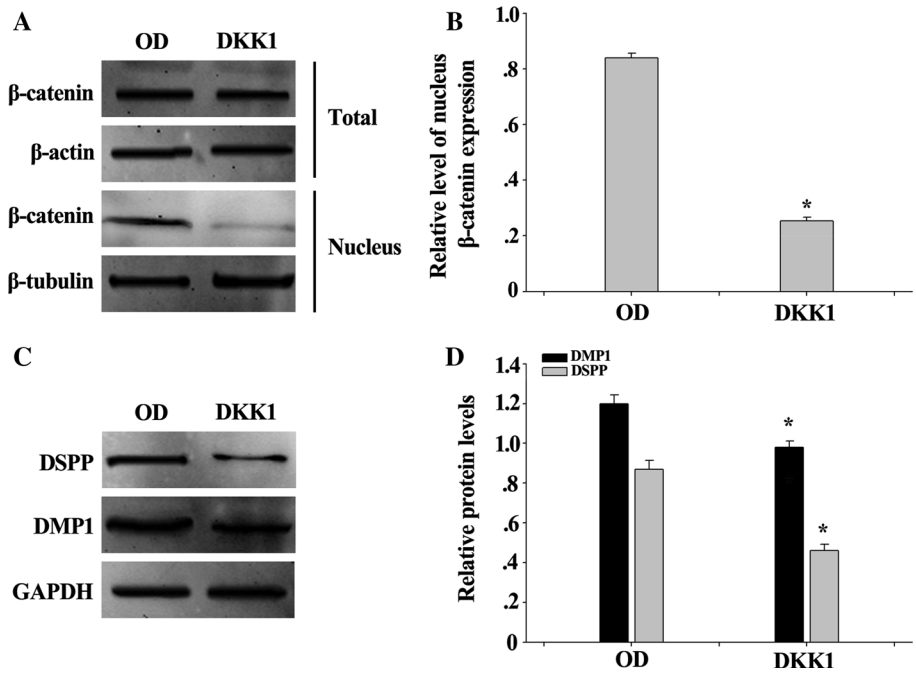

Fig. 5 Suppression of β-catenin by DKK-1 inhibits odontogenic differentiation. **a** DPSCs were cultured in odontogenic differentiation medium for 14 days after DKK-1 treatment. The total and nuclear β-catenin levels were analyzed by Western blot. β-actin was used as the internal control for cytoplasmic proteins. **b** Quantification of nuclear β-catenin protein level. $*P < 0.05$. **c** The expression of DSPP and DMP1 proteins were analyzed by Western blot. **d** Quantification of DSPP and DMP1 protein levels after DKK-1 treatment. $*P < 0.05$

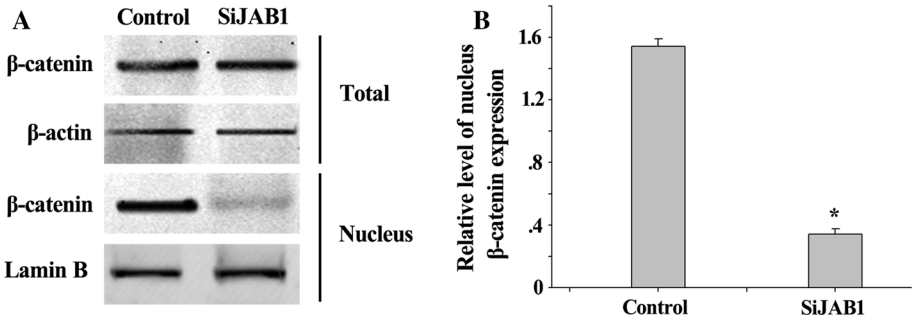

Fig. 6 Suppression of JAB1 inhibits the expression of nuclear β-catenin in DPSCs. **a** siRNA was used to silence JAB1 expression. The total and nuclear β-catenin levels were detected by Western blot. β-actin was used as the internal control for cytoplasmic proteins. **b** Quantification of nuclear β-catenin protein levels. $*P < 0.05$

Activation of Wnt/β-catenin signaling pathway in odontogenic differentiation of DPSCs

To examine the expression of Wnt/β-catenin signaling on odontogenic differentiation of DPSCs, we examined the expression levels of GSK-3β and β-catenin, and found that nuclear β-catenin expression was significantly higher in the differentiation group (Fig. 4a, b). Western blot showed that the expression of nuclear β-catenin was significantly lower in the normal group as compared to the differentiation group (Fig. 4b). Furthermore, immunocytochemistry showed higher nuclear β-catenin staining intensity in the differentiation group as compared to the control group (Fig. 4c, d). These results indicated that nuclear translocation of β-catenin could be increased in the differentiation group and that activation of Wnt/β-catenin signaling may be promoted by the differentiation process.

Suppression of β-catenin by DKK-1 inhibits odontogenic differentiation

DPSCs were cultured in odontogenic differentiation medium, and treated with 100 ng/mL DKK-1 to inhibit Wnt/β-catenin. After DKK-1 treatment, the nuclear expression of β-catenin was significant decreased (Fig. 5a, b). Western blot showed that the expression of DSPP and DMP1 proteins also decreased (Fig. 5c, d). These findings suggested that inhibition of Wnt/β-catenin inhibited odontogenic differentiation.

Suppression of JAB1 inhibits the expression of nuclear β-catenin in DPSCs

To determine the effect of JAB1 in Wnt/β-catenin signaling pathway, we used siRNA to silence JAB1 expression.

Western blot showed that the expression of nuclear β-catenin was significantly lower in the siRNA group as compared to the control group (Fig. 6a, b). These results showed that siRNA-mediated silencing of JAB1 expression could inhibit odontogenic differentiation of DPSCs via Wnt/β-catenin signaling pathway.

Discussion

Our study demonstrated for the first time that JAB1 increased the expression of odontogenic markers DSPP and DMP1, and had a positive effect on odontogenic differentiation of DPSCs. Furthermore, Wnt/β-catenin signaling plays an essential role in controlling the odontogenic differentiation of DPSCs.

Recently, we reported several molecular target differentiation factors of DPSCs that may have clinical implications for regenerative endodontics (Feng et al. 2015; Xing et al. 2015). JAB1 is the fifth component of the COP9 signalosome (CSN) complex (COPS5) that is involved in various cellular and developmental processes (Wei and Deng 2003). Herein, we investigated the influence of JAB1 on the odontogenic differentiation of DPSCs. JAB1 protein level was significantly increased after 14 days of odontogenic induction. To assess the role of JAB1 in the odontogenic differentiation of DPSCs, we tested the effects of JAB1 siRNA on the expression of differentiation markers, which showed that silencing of JAB1 significantly decreased the levels of DSPP and DMP1, and inhibited the odontogenic differentiation of DPSCs.

Multiple signaling pathways contribute to the odontogenic differentiation of DPSCs. Wnt/β-catenin signaling was shown to play an essential role in controlling osteoblast and chondrocyte differentiation of MSCs (Hunter et al. 2015). A recent study found that Wnt/β-catenin signaling pathway is partially responsible for Berberine-induced odontogenic differentiation of MSCs in vitro (Tao et al. 2016). Furthermore, JAB1 is thought to be critical for the regulation of β-catenin levels and the associated Wnt signaling in colorectal cancer cells (Schutz et al. 2012). So we determined whether increased expression of JAB1 led to the activation of Wnt/β-catenin signaling pathway. During odontogenic differentiation of DPSCs, we found that the expression of nuclear β-catenin was significantly increased. siRNA-mediated silencing of JAB1 expression could reduce the expression of nuclear β-catenin. Furthermore, the expression of DSPP and DMP1 was significantly decreased after JAB1 knockdown. Taken together, these results showed that JAB1 enhanced odontogenic differentiation of DPSCs via Wnt/β-catenin signaling pathway.

In conclusion, we successfully detected the expression of JAB1 and confirmed its requirement during the odontogenic differentiation of DPSCs. Further work is necessary to determine how JAB1 regulates the differentiation of odontoblasts and find the genes that interact with JAB1.

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